

REMARKS

Claims 1-18 were pending. Claims 2, 3, 11 and 12 have been withdrawn from consideration. Claims 1 and 10 are amended. No new matter is added. In view of the following remarks, Applicants respectfully request reconsideration of the rejections.

The Office Action states that the Restriction Requirement previously set forth is made final. Applicants reserve the right set forth in 37 C.F.R. 1.114 and M.P.E.P. 818.03(c) to petition the Commissioner for review of the requirement, which petition may be deferred until after final action on or allowance of claims to the invention elected.

The reference to priority in the specification has been appropriately updated.

Claims 1, 4, 5-10 and 13-18 have been rejected under 35 U.S.C. 112, second paragraph, as indefinite in the recitation of the term "integrin-linked kinase". Applicants respectfully submit that the term is known in the art, and properly identifies the protein in question. For example, the widely used scientific databases of the National Library of Medicine and National Center for Biotechnology Information (accessible at <http://www.ncbi.nlm.nih.gov/>), which include the Online Mendelian Inheritance of Man (OMIM) and Genbank, refer to the kinase identified in the present application as "integrin-linked kinase, ILK, or the secondary name p59. See, for example, (Online Mendelian Inheritance in Man, OMIM™. Johns Hopkins University, Baltimore, MD. MIM Number: {602366}: {2/18/1998}: World Wide Web URL: <http://www.ncbi.nlm.nih.gov/omim/> (602366). The nomenclature is consistently used in the literature, for example in Genbank (accession number NM_004517); in the Genome Database; and in Pubmed (for example, see White *et al.* (2001) ONCOGENE, 20(48): 7064-7072.

Applicants respectfully submit that the use of term "integrin-linked kinase" provides a clear definition of the intended subject matter to one of skill in the art. Withdrawal of the rejection is requested.

Claims 1, 4-7, 10, and 13-16 have been rejected under 35 U.S.C. 102 as anticipated by Norman *et al.* (1996) *J. Med. Chem.* **39**:1106-1111. Applicants respectfully submit the presently claimed invention is not anticipated by the cited art.

Norman *et al.* describe wortmannin as an inhibitor of PI 3-kinase, which also displays cytotoxicity towards human tumor cell lines (page 1108, last paragraph). The reference discusses

the antitumour activity of various wortmannin analogs. However, the reference fails to teach an effect of wortmannin on inflammation, as set forth in the present claims, and therefore cannot anticipate the present claims. In view of the above remarks, withdrawal of the rejection is requested.

Claims 1, 4, 5-10 and 13-18 have been rejected under 35 U.S.C. 112, first paragraph. The Office Action states "assertions . . . indicate that due to the non-specific inhibitory effects of wortmannin and LY294002, further research is required in the art to employ other, more specific inhibitors of integrin linked kinase", and that the specification does not provide particular guidance or particular direction for the inhibition or prevention of inflammation in a host. Applicants respectfully submit that the presently claimed invention is enabled, and could be practiced by one of ordinary skill in the art without undue experimentation.

The Office Action refers to the lack of specificity of wortmannin and LY294002 as being indicative that they are not useful inhibitors *in vivo*, and that undue experimentation would be required to determine useful inhibitors based on our disclosure. The Office Action asserts judgements on the medical applicability of the compounds based on assumptions made about specificity. However, non-specific activity is not indicative that a compound is not a useful clinical entity. For example, the compounds ZD6474 (currently in Phase I Clinical trials), and SU6668 (in Phase II trials) are representatives of multitarget tyrosine kinase inhibitors (see Dreves *et al.* (2003) Curr Drug Targets 4(2):113-21, abstract attached), and are said to be particularly interesting in their potential for chronic therapy (see Glade-Bender *et al.* (2003) Expert Opin Biol Ther 3(2):263-76, article attached).

In addition, at the time the present patent application was filed, Applicants were working on developing ILK-specific inhibitors. These inhibitors are the subject of several issued US patents. For example, see U.S. Patent nos. 6,214,813; 6,436,914; and 6,420,400. A number of applications describing ILK inhibitors are also pending, for example, United States Patent Application 20030060453, Zhang *et al.*, March 27, 2003.

The purported lack of correlation between the assessment of integrin linked kinase activation by insulin of IEC-18 cells would have been clear to a person skilled in the art. Intestinal epithelial cells are a first barrier to a number of sources of infection, and subsequently, these cells produce a variety of chemokines in response to bacterial infection or proinflammatory cytokines. These chemokines act as potent leukocyte activators and chemoattractants *in vivo*. For example, interleukin-1 (IL-1), an important mediator of inflammation, is expressed on intestinal epithelial cells (Sutherland *et al.* (1994) Am J Physiol 266:C1198-203). Further, Waterhouse and Stadnyk (1999)

Cell Immunol 193:1-8 have shown that intestinal epithelial cells produce IL-1 β *in vivo*, and this action precedes most inflammation. The use of IEC-18 as an *in vitro* model for testing anti-inflammatory potential for ILK inhibitors was therefore a rational choice.

Additional examples demonstrating the anti-inflammatory effect of ILK inhibitors may be found in U.S. Patent Application 20020155179, Dedhar *et al.*, October 24, 2002. The anti-inflammatory activity of the anti-ILK compound MC-5 was demonstrated in an acute mouse ear-swelling edema model. To induce this inflammatory experimental condition, mice are treated topically on the surface of an ear with tetra phorbol ester (TPA). Application of TPA in such a manner produces a rapid increase in ear thickness caused by fluid buildup and the infiltration of the tissue by inflammatory cells. Different doses of MC-5 were given orally at the same time as an active amount of TPA. Ear measurements performed 6 hours after these treatments showed that a dose of MC-5 of 200 mg/kg almost completely prevented the increase in ear swelling stimulated by TPA. The effect of this dose of MC-5 on this response was comparable to that produced by dexamethasone, a well-characterized and potent anti-inflammatory agent. Thus, a compound that is known to inhibit the activity of ILK *in vitro* can also affect the development of symptoms of an experimental inflammatory skin condition *in vivo*.

A further example demonstrates that an anti-ILK compound inhibits influx of neutrophils into a site of inflammation. Administration of certain pro-inflammatory agents, such as zymosan, into the peritoneal cavity of mice elicits a rapid influx of neutrophils into this region. The migration of these cells into the peritoneal cavity requires the coordinate interaction of cytokines, chemokines and cell adhesion molecules. Such a system is used to evaluate the action of compounds with potential for modifying the migration of cells in response to pro-inflammatory stimuli.

When zymosan was administered to mice, peritoneal cavity neutrophil numbers increased by approximately 4-fold within 4 hours. However, if MC-5 was given orally at 200 mg/kg at the time of zymosan administration cells numbers within the peritoneal cavity were equivalent to those of animals that received a saline control solvent 4 hours before. Thus, a compound with anti-ILK activity can affect the influx of cells into a tissue following the delivery of a strong pro-inflammatory signal.

Applicants have provided guidance for the practice of the claimed invention. Administration instructions are found in paragraphs 52 and 56 – 69 of the present application. It is common for companies to file patent applications prior to a final dosage form of a drug being established. It is established in case law that determination of a dosage form requires no more than routine experimentation.

One skilled in the art would have little difficulty in coming up with a useful formulation for ILK inhibitors. In *Hu et al.* (2002), LY294002 was administered in DMSO and PBS by intraperitoneal injection at 100 mg/kg three times weekly. In U.S. Patent No. 5,491,242, staurosporines of the same family are formulated for oral administration using carboxymethylcellulose. In U.S. Patent No. 5,736,542, staurosporines are formulated in solid saturated polyalkylene glycol glyceride. Wortmannin is soluble in DMSO and methanol, but would most likely be delivered in a lipid-based formulation. In US Patent No. 5,468,773, several oral formulations and inhaled formulations are described in detail.

The law regarding enablement of inventions is clear: “[t]he test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation.”¹

To aid in determinations of enablement, courts have identified factors for consideration, including: (a) the quantity of experimentation necessary; (b) the amount of direction or guidance presented; (c) the presence or absence of working examples; (d) the nature of the invention; (e) the state of the prior art; (f) the relative skill of those in the art; (g) the predictability or unpredictability of the art; and (h) the breadth of the claims.²

The instant specification teaches the identification of integrin-linked kinase, specific compounds that inhibit the enzyme, and methods of screening for inhibitory agents, and methods of administration.

Applicants respectfully submit that the specification and the amended claims, coupled with the information known in the art, would enable one of skill in the art to use the invention without undue experimentation. Relevant enablement factors are discussed in detail below.

The courts have clearly taught that the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. For example, see MPEP §2164.01.³

¹ *United States v. Telectronics, Inc.*, 8 USPQ 2d 1217, 1233 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1046 (1989). See also *Genentech, Inc. v. Novo Nordisk*, 42 USPQ 2d 1001 (Fed. Cir. 1997), *cert. denied*, 522 U.S. 963 (1997); *Scripps Clinic and Research Foundation v. Genentech, Inc.*, 18 USPQ 2d 1001 (Fed. Cir. 1991).

² *Ex Parte Forman*, 230 USPQ 546, 547 (Bd. Pat. App. & Interf. 1986); and, *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).

³ See also *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd sub nom.*, *Massachusetts Institute of Technology v. A.B. Fortia*, 227 USPQ 428 (Fed. Cir. 1985).

As the court explained⁴:

"[A] considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed."

Practitioners in the chemical and molecular biology arts frequently engage in extensive modification of reaction conditions and complex and lengthy experimentation where many factors must be varied to succeed in performing an experiment or in producing a desired result. The Federal Circuit has found that such extensive experimentation is not undue in the molecular biology arts. For example, the court concluded that extensive screening experiments, while being voluminous, were not undue in view of the art, which routinely performs such long experiments.⁵

The claimed methods utilize specific compounds identified in the specification, or compounds that were identified by the screening methods provided in the specification. One need only optimize dose and formulation for practice of the invention. Since such experiments are empirical in nature, no undue experimentation is required. In other words, the only experimentation that may be required to enable the claimed invention are those experiments to determine the appropriate dose of a certain activity, and since this only requires a routine assay to determine the active variants, no undue experimentation is necessary.

Compliance with the enablement requirement under Section 35 U.S.C. §112, first paragraph does not require or mandate that a specific example be disclosed. The specification need not contain a working example if the invention is otherwise disclosed in such a manner that one skilled in the art would be able to practice the invention without undue experimentation.⁶ Furthermore, "Nothing more than objective enablement is required, and therefore it is irrelevant whether [a] teaching is provided through broad terminology or illustrative examples."⁷ As discussed above, several specific examples of inhibitors have been provided.

The relevant ordinarily skilled artisan is generally a skilled laboratory technician with the equivalent of a doctoral degree in molecular biology techniques. Furthermore, such technicians are required to keep abreast of the latest technology through continuing education and reading of scientific journal articles. As such, the skill level of those developing and using methods for manipulating performing cell-based assays is high.

In sum, the amount of experimentation required to inhibit or prevent inflammation using compounds that specifically inhibit integrin-linked kinase, as identified by Applicants, would not be

⁴ *In re Wands* 8 USPQ 2d at 1404

⁵ *Hybritech v. Monoclonal Antibodies, Inc.* 231 USPQ 81 (Fed. Cir. 1986)

⁶ *In re Borkowski*, 164 USPQ at 645.

⁷ *In re Robins* 166 USPQ 552 at 555 (CCPA 1970).

undue because a) examples of inhibitors are provided, b) guidance is given on how to screen for additional inhibitors, and c) one of skill in the art would be able to perform the experiments as a matter of routine to determine the optimal dosage.

The specification therefore provides sufficient enablement such that one of ordinary skill in the art would be able to practice the invention without undue experimentation. In view of the above amendments and remarks, withdrawal of the rejection is requested.

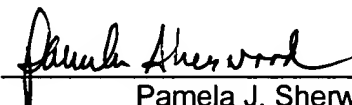
CONCLUSION

Applicants submit that all of the claims are now in condition for allowance, which action is requested. If the Examiner finds that a Telephone Conference would expedite the prosecution of this application, she is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any other fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number KINE-001CON2.

Respectfully submitted,

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Receptor tyrosine kinases: the main targets for new anticancer therapy.

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Because conventional chemotherapy is not specific for cancer cells leading to toxic side effects there is a need for novel agents with high grade antitumor specificity. The major prerequisite to develop such drugs is to understand the targets that these agents should attack. In recent years a number of promising new anticancer drugs have been developed which target intracellular pathways or extracellular cell molecules. The clinically most effective compounds function as tyrosine kinase inhibitors. In the past, various tyrosine kinase receptors have been identified as regulators of tumor or tumor vessel growth. Having shown their expression characteristics in different tumor entities, specific inhibitors of the ATP binding sites of these receptors or antibodies were developed and entered clinical trials. The pathognomonic role of the tyrosine kinase defines the way of action of the inhibiting drug, whereas the amount of expression in tumor tissue defines the rationale to use the inhibitor to treat a specific protein. The future will define indications for such drugs by tumor kinase profiles instead of tumor entities. Gleevec, inhibiting the BCR-ABL tyrosine kinase; Iressa, inhibiting the EGF-receptor tyrosine kinase; Herceptin, inhibiting the Her2/neu tyrosine kinase and PTK787/ZK222584, inhibiting the VEGF-receptor tyrosine kinase will be discussed as representatives of selective tyrosine kinase inhibitors whereas ZD6474 and SU6668 will be discussed as representatives of multitarget tyrosine kinase inhibitors.

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Cytokines & Chemokines

VEGF blocking therapy in the treatment of cancer

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It is widely accepted that tumour growth beyond a few cubic millimetres cannot occur without the induction of a new vascular supply. Inhibiting the development of new blood vessels (angiogenesis) is a potential approach to cancer therapy that has attracted interest in recent years. In theory, this approach should be relatively selective for tumour cells. The endothelial cells which form new vascular networks in tumours are responding to angiogenic stimuli produced by the tumour, but are themselves genetically normal. Endothelium in normal tissue, by contrast, is usually quiescent. Vascular endothelial growth factor (VEGF) is the best-characterised pro-angiogenic factor. It is virtually ubiquitous in human tumours, and higher levels have been correlated with more aggressive disease. Effective blockade of the VEGF pathway has been demonstrated with multiple agents: neutralising antibody, receptor tyrosine kinase inhibitors, and ribozyme or antisense molecules targeting expression. Promising preclinical data document the potential of these agents for tumour growth inhibition and even tumour regression, yet translation of novel therapeutics targeting the VEGF pathway to the clinic has proved a substantial challenge in itself. While showing clear evidence of antitumour activity over a broad spectrum of experimental tumours, the proper selection, dose, timing and sequence of anti-VEGF treatment in human cancer is not at all obvious. Classic Phase I dose escalation trial design may need to be modified, as higher doses may not be optimal in all patients or for all tumours. In addition, alternate or secondary biological end points (e.g., non-progression) may be needed for early phase studies to document true activity, so as not to abandon effective agents. Recent studies of the neutralising antibody bevacizumab, and small molecule tyrosine kinase inhibitor SU5416, demonstrate that, while unlikely to be effective as monotherapy, incorporation of VEGF blockade into cytotoxic regimens may increase overall response rates. However, incorporation may also produce new toxicities, including thromboembolic complications and bleeding. Newer oral agents, such as SU6668, SU11248, PTK787/ZK222584 and ZD6474, are particularly interesting for their potential for chronic therapy. Future clinical trials are likely to build on past experience with stricter entry criteria, supportive care guidelines and the use of surrogate markers.

Keywords: angiogenesis, angiozyme, avastin, bevacizumab, CEP-7055, CP-547,632, HuMV833, IMC-1C11, PTK787/ZK 222584, SU5416, SU6668, SU11248, vatalanib, VEGF, VEGF receptors, VEGF-Trap, ZD6474

Expert Opin. Biol. Ther. (2003) 3(2):263-276

1. Introduction

Angiogenesis, the process of new blood vessel formation, is an essential step in tumour growth and metastasis. A growing understanding of this critical phase in tumour progression has prompted extensive efforts at developing antiangiogenic

agents as novel cancer therapeutics. Vascular endothelial growth factor (VEGF) is the best-characterised pro-angiogenic cytokine, and disrupting VEGF signalling is, to date, the best-validated therapeutic approach to inhibiting angiogenesis. The VEGF pathway is a key mediator of vascular development in embryogenesis and physiologic growth, but also plays an important role in pathologic angiogenesis. This article will review the role of VEGF in tumour angiogenesis and examines current approaches to blocking the VEGF pathway.

2. Vascular endothelial growth factor

VEGF was initially identified by Dvorak in 1983 as a tumour-derived factor capable of increasing vascular permeability (hence its original name of vascular permeability factor) [1]. It was subsequently purified and sequenced by Ferrara in 1989, and identified as a potent mitogen for endothelial cells [2]. VEGF can elicit a pronounced angiogenic response *in vivo* and is a survival factor for endothelial cells both *in vitro* and *in vivo* [3].

VEGF is critical for normal vessel development in the embryo, where it promotes the differentiation, proliferation and survival of endothelial cells, and elaboration of the vascular tree. The importance of VEGF is highlighted by the finding that the loss of a single VEGF allele is lethal in the mouse embryo during days 11 and 12 [4,5]. In the adult, VEGF is upregulated in a variety of normal and pathological processes associated with increased vascular permeability and angiogenesis [3].

There are numerous isoforms of VEGF which are generated by alternative exon splicing of a single gene consisting of eight exons [6]. The principal VEGF isoforms are VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆, with less frequent splice variants VEGF₁₄₅ and VEGF₁₈₃ [3,7]. Recently, an inhibitory splice variant, VEGF_{165b}, has also been described, which is downregulated in renal cell carcinoma [8]. VEGF₁₂₁ lacks a heparin-binding region and is a freely diffusible protein. VEGF₁₆₅ is also soluble but contains a heparin-binding motif. Thus, VEGF₁₆₅ is significantly bound to cell surface and extracellular matrix (ECM) heparin sulfates. VEGF₁₈₉ and VEGF₂₀₆ bind heparin with very high affinity and are sequestered in the ECM. Due, in part, to differential binding to heparin, the VEGF isoforms demonstrate different angiogenic characteristics [9,10]. In human melanoma xenografts, expression of VEGF₁₂₁ or VEGF₁₆₅, but not VEGF₁₈₉, is tumorigenic [10]. There were also striking isoform-specific differences in tumour vasculature: tumours overexpressing VEGF₁₂₁ are poorly vascularised and necrotic, while tumours overexpressing VEGF₁₆₅ display dense, ramified vessel networks.

In addition to the different VEGF isoforms, there is a family of VEGF-related angiogenic growth factors: VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF) [3,7]. The precise functions of many of these VEGF-related ligands are not known or are ambiguous. However, the activity of each ligand is likely to depend, in part, on differential affinity for the three signalling VEGF receptors, VEGFR-1 (*Flt-1*), VEGFR-2 (*K1/K2/KDR*) and VEGFR-3 (*FLT4*).

VEGF-A binds to both VEGFR-1 and VEGFR-2. PlGF and VEGF-B bind exclusively to VEGFR-1. VEGF-C and -D bind to VEGFR-2 and -3, and are mitogens for both vascular and lymphatic endothelial cells. VEGF-E is encoded by the parapoxvirus, Orf virus, and can mediate angiogenesis via signalling through VEGFR-2.

VEGFR-2 appears to be the principal receptor by which VEGF exerts its angiogenic effects [3]. Using receptor-selective VEGF mutants, Gille *et al.* have demonstrated that VEGF-mediated migration, angiogenesis and vascular permeability is the function solely of VEGFR-2 [11]. Antibodies specific for VEGFR-2 have also demonstrated the essential role of this receptor for angiogenesis [12-14].

The role of VEGFR-1 has only recently become appreciated. Hiratsuka *et al.* had previously shown that VEGFR-1 without a tyrosine kinase domain is sufficient to allow embryonic development with normal angiogenesis, and that the receptor tyrosine kinase plays a main biological role as a ligand-binding molecule [15]. Recently, however, the importance of VEGFR-1 in pathological angiogenesis has been demonstrated using anti-VEGFR-1 antibodies [16,17] and by antisense-mediated downregulation of VEGFR-1 by Angiozyme® (Ribozyme Pharmaceuticals, Inc.) [18], both of which inhibit tumour growth. In addition, both VEGFR-1 and -2 appear to be critical for the recruitment of bone marrow-derived endothelial precursor cells to newly formed tumour vasculature [19]. Lastly, VEGFR-1 signalling may be involved in other processes in tumour pathogenesis. Hiratsuka *et al.* have recently shown that the matrix metalloprotease (MMP)9 is specifically induced in premetastatic lung endothelial cells and macrophages by distant primary tumours via VEGFR-1, and that it significantly promotes lung metastasis [20]. They also show that suppression of MMP9 induction by deletion of either VEGFR-1TK or MMP9 markedly reduced lung metastasis. Therefore, although most efforts at blocking VEGF receptors have focused on inhibiting VEGFR-2, it is possible that an agent also disrupting VEGFR-1 activation might have a greater ability to inhibit tumour pathogenesis [21,22].

VEGF isoforms that have a heparin-binding site can also bind to the semaphorin receptors neuropilin-1 and neuropilin-2 [23]. Their exact role in angiogenesis is not clear, but recent evidence has shown that neuropilin-1 is required for vascular development and is a mediator of VEGF-dependent angiogenesis in zebrafish [24]. Neuropilin-1 can also modulate binding to VEGFR-2 and subsequent bioactivity [25]. Neuropilins are expressed in various human cancers, including neuroblastoma [26] and breast carcinoma [27].

In addition to their roles in angiogenesis, VEGF and its receptors are also involved in the regulation of haematopoiesis [28]. VEGF and its receptors are expressed in haematopoietic stem cells (HSC) and in malignancies of haematopoiesis (see below). Recently, Gerber and co-workers have shown that ablation of the VEGF gene in adult mice reduces the survival, colony formation and *in vivo* repopulation rates of HSCs [32]. In addition, they show the

presence of an internal autocrine loop mechanism, in which small-molecule inhibitors of VEGFR, but not an extracellular soluble VEGFR-1, reduced colony formation of HSCs. Hattori *et al.* have also demonstrated that inhibition of VEGFR-1, but not VEGFR-2, blocked HSC cell cycling, differentiation and haematopoietic recovery after bone marrow suppression, resulting in the demise of the treated mice [33]. PlGF, which signals through VEGFR-1, restored early and late phases of haematopoiesis following bone marrow suppression. These results suggest that VEGF blockade therapy, if combined with chemotherapy, may result in prolongation of bone marrow suppression.

VEGF is also involved in pathological conditions such as rheumatoid arthritis [34], asthma [35] and psoriasis [36]. In proliferative diabetic retinopathy and age-related macular degeneration (AMD), ocular neovascularisation (AMD) can be inhibited by VEGF blocking agents [37,38]. This has led to clinical trials for AMD with an antigen-binding fragment of a recombinant humanised mAb to VEGF (rhFab VEGF/ranibizumab; Genentech), which has shown preliminary positive Phase Ib/II results [201].

3. VEGF in cancer

VEGF is ubiquitously expressed in almost all human tumours studied to date. High levels of VEGF had been found to correlate with more advanced stages or with a worse prognosis in tumours of the bladder [39], brain [40,41], breast [42,43], colon [44], lung [45-47], ovary [48,49], and in neuroblastoma [50], renal cell carcinoma [51] and squamous cell carcinomas of the neck [52]. Therefore, interference with VEGF-mediated angiogenesis appears to hold promise as a general approach to controlling the growth and metastatic spread of human tumours.

There has also been increasing evidence that certain tumours may express, in addition to the VEGF ligand, VEGF receptors, thus potentially creating an autocrine growth factor loop. This has been described for breast carcinoma [27], melanoma [53], mesothelioma [54], ovarian carcinoma [53,55] and prostate cancer [56]. Bachelder *et al.* report that VEGF is a requisite autocrine factor for breast carcinoma invasion *in vitro* and that neuropilin-1, but not VEGFR-1, is essential for this function [57]. VEGF regulates expression of the chemokine receptor CXCR4, and this VEGF target is required for invasion but not for tumour cell survival.

While there is much evidence for its critical role in solid tumour angiogenesis, the importance of VEGF in haematological malignancies has only more recently been appreciated. VEGF is implicated in the induction of angiogenesis in peripheral T cell lymphomas, Castleman's disease, Hodgkin's disease [58] and multiple myeloma [59]. Aguayo *et al.* have shown that elevated levels of VEGF are associated with reduced survival in patients with acute myelogenous leukaemia (AML), but not for patients with myelodysplastic syndrome (MDS) [60]. The exact function of VEGF in haematologic malignancies is not known. However, it appears

to promote bone marrow angiogenesis and may play additional roles. Dias *et al.* have shown that leukaemias not only produce VEGF, but also selectively express functional VEGFR-1 and VEGFR-2, resulting in the generation of an autocrine loop [61]. The same group have also shown, using a mouse model of human leukaemia, that long-term remission can be achieved only if mice are treated with mAbs against both murine and human VEGFR-2 [13]. This suggests that blocking both paracrine and autocrine VEGF/VEGFR-2 angiogenic loops may result in long-term remissions of VEGF-producing, VEGFR-expressing leukaemias. These studies indicate that anti-VEGF blocking agents may be of value in treating not only solid tumours that form new vascular networks, but haematologic malignancies as well [62].

VEGF may also be involved in the 'angiogenic switch' required for tumour progression [63]. Fang *et al.* have shown that hypoxia-inducible factor (HIF)-1 α -mediated increase in VEGF is involved in the angiogenic switch, by examining the avascular and vascular growth phases of tumours [64]. Hanahan and colleagues have shown that VEGF is critical for the angiogenic switch, through use of the VEGF blocker SU5416 (semaxanib; SUGEN/Pharmacia) [65]. In addition, using a RIP1-Tag2 mouse model of pancreatic islet carcinoma, with an islet beta cell specific knockout of VEGF, they demonstrated that transition to the angiogenic phenotype and tumour growth were severely disrupted, as was the neovasculature [66].

In addition to its pro-angiogenic functions, the permeability-inducing properties of VEGF are of both pathological and clinical importance. VEGF is found in malignant effusions and ascites from a variety of tumour types [67,68]. Studies by Dvorak and colleagues [69,70] and Luo *et al.* [71] demonstrated that tumour cells expressing high levels of VEGF induce ascites, which can be blocked by antibodies to VEGF. Therefore, targeting the VEGF pathway may provide an additional benefit to cancer patients by reducing symptoms caused by the paraneoplastic secretion of VEGF.

4. Targeting the VEGF pathway

The VEGF receptor tyrosine kinase family plays an essential role in tumour angiogenesis and, thus, its members are attractive targets for cancer therapy. Multiple strategies to block the VEGF pathway have been shown to be efficacious in inhibiting tumour angiogenesis in preclinical animal models. Strategies include:

- targeting the ligand with anti-VEGF antibodies [72-74], soluble forms of VEGF receptors [75,76] or RNA-based aptamers which bind to VEGF [77]
- targeting VEGF receptors with anti-VEGFR antibodies [78] or inhibiting the tyrosine kinase activity [79-81]
- ribozymes, which downregulate VEGF receptor function by specifically cleaving the mRNAs for VEGF receptors [18]
- targeting endothelial cells via the receptors with a toxin coupled to VEGF [82]
- antisense therapy against VEGF or VEGFR [83-87]

Table 1. VEGF blocking agents in clinical trials.

Agent	Phase	Sponsor	Activity
Bevacizumab	II/III	Genentech	Anti-VEGF antibody
HuMV833	I	Toagosei & Protein Design Labs	Anti-VEGF antibody
IMC-1C11	I	ImClone Systems	Anti-VEGFR-2 antibody
VEGF-Trap	I	Regeneron	Soluble VEGFR1/R2
SU5416	II/III*	SUGEN/Pharmacia	VEGFR inhibitor
SU6668	I	SUGEN/Pharmacia	VEGFR/PDGFR/FGFR inhibitor
SU11248	I	SUGEN/Pharmacia	VEGFR/PDGFR/c-Kit inhibitor
PTK787/ZK 222584	I	Novartis/Schering	VEGFR-2 inhibitor
ZD6474	I	AstraZeneca	VEGFR-2 inhibitor
CP-547,632	I	Pfizer/OSI	VEGFR inhibitor
CEP-7055	I	Cephalon	VEGFR inhibitor
Angiozyme®	II	Ribozyme Pharmaceuticals	Anti-VEGFR-1 ribozyme

* Development discontinued.

FGFR: Fibroblast growth factor receptor; PDGFR: Platelet-derived growth factor receptor; VEGF: Vascular endothelial growth factor; VEGFR: VEGF receptor.

Despite this array of promising preclinical data, the translation to effective human cancer therapy has been more difficult than anticipated. Nonetheless, important strides have been made, with a growing number of agents presently in clinical trials (Table 1). To date, there have been several large-scale Phase I/II clinical trials of different VEGF blocking therapies, establishing dose, toxicity profile and lead indications. Recently, a few compounds have reached the Phase III stage and have even completed accrual, although the data has not yet been thoroughly analysed.

4.1 Bevacizumab

Recombinant humanised monoclonal neutralising antibodies to VEGF were among the first agents available for clinical trials, with the furthest along the pipeline being bevacizumab (BV) (rhumaB-VEGF A4.6.1/Avastin™; Genentech). The origins of BV date back to 1993, when Ferrara and colleagues demonstrated that VEGF was critical for tumour growth and angiogenesis by use of a murine mAb specific to human VEGF (muMab VEGF A4.6.1) [72]. This antibody was then humanised by site directed mutagenesis of the human framework (rhumaB VEGF A4.6.1), with retention of high affinity binding ($K_d = 1.8$ nM) to VEGF [73]. The efficacy of A4.6.1 to inhibit angiogenesis and tumour growth has been widely demonstrated in numerous tumour models, including breast [88], prostate [89], rhabdomyosarcoma [90] and Wilms' tumour [91]. A4.6.1 has also been shown to be effective at inhibiting ascites [92].

The first Phase I trial of BV was conducted in 25 adults with a variety of solid tumours, to assess the pharmacokinetics and safety of single agent therapy. Patients received doses of 0.1 – 10 mg/kg i.v., with no common toxicity criteria (CTC) grade 3 – 4 adverse events deemed definitively related to therapy. Grade 1 – 2 reactions were typical of those seen

with antibody infusion and included headache, fever, nausea, vomiting, arthralgia and rash. Most patients developed transient, mild systolic hypertension. There were four episodes of tumour-related bleeding. Two were considered serious and included an intracranial bleed in a patient with unrecognised CNS metastasis. Although no objective responses were noted, there were two minor responses and 12 of 23 patients achieved stabilisation of disease over the 70-day study period. Free serum VEGF levels were reduced to below detectable levels after a single dose of BV ≥ 3 mg/kg. At doses ≥ 0.3 mg/kg, clearance of BV was found to be linear and consistent, with a favourable half-life of 21 days [93].

Encouraging therapeutic results with BV as a single agent have come from a prospective three-arm, double-blind trial comparing placebo (P), 3 mg/kg (low dose [LD]) and 10 mg/kg (high dose [HD]) antibody given every 2 weeks in patients with renal cell carcinoma (RCC) [94]. RCC may be particularly sensitive to VEGF blockade, since the majority of RCC have mutations in the von Hippel-Lindau (VHL) tumour-suppressor gene that leads to the oversecretion of VEGF [95]. After 110 patients were randomised (38 P, 35 LD and 37 HD), interim analysis showed a highly significant prolongation of time-to-progression of HD antibody versus P (hazards ratio = 2.3, $p = 0.001$), satisfying early stopping criteria. The difference between LD and P was of borderline significance. There were three partial responses, all to HD antibody (response rate = 8%).

Additional Phase I/II studies of BV in adults identified promising target indications as breast, colorectal, kidney, non-small cell lung cancer (NSCLC) and, to a lesser extent, prostate cancer with clinical activity manifested most often as increased time-to-progression, rather than objective clinical response. For those patients with clinical benefit, there have been 28 who have gone on to receive BV for ≥ 1 year on an

extension study. This long-term therapy was generally well-tolerated, with median time to progression of 13.7 months. Deep vein thromboses developed in five patients (18%). Other significant adverse events were hypertension, proteinuria and gastrointestinal bleeding in two patients with colon cancer [96]. It is interesting to note there have been no reports of poor wound healing or menstrual abnormalities.

In order to achieve tumour regression, many studies were designed to examine the activity of BV in combination with standard cytotoxic agents [97-101]. These have shown promise for synergistic efficacy with minimal additive toxicity. Grade 3 - 4 toxicities have been predominately attributable to the cytotoxic agents, with two notable and likely specific exceptions: a propensity for deep vein thrombosis (DVT) and a possible increased risk of life-threatening haemorrhage. In an NSCLC trial, 99 patients were randomised to receive either carboplatin/paclitaxel alone, with LD BV (7.5 mg/kg every 3 weeks) or with HD BV (15 mg/kg every 3 weeks). Unexpectedly, there were life-threatening pulmonary haemorrhages in 6 of 66 subjects assigned to one of the two experimental arms, resulting in four study-related deaths [97]. Subsequent analysis identified squamous histology and BV treatment as statistically significant risk factors, with the additional suggestion that centrally-located, cavitary tumours may be at greatest risk of bleeding [98]. Nonetheless, the study suggested a very encouraging benefit to HD BV therapy in the overall response rate, time-to-progression and median survival time, which may exceed the incremental risk [99,100]. Larger Phase II or III trials will need to establish strict entry criteria and careful monitoring for clinically significant haemoptysis in order to move the development of BV forward for primary lung or pulmonary metastatic diseases.

The Eastern Cooperative Oncology Group (ECOG) has recently reported on a relatively large, multi-centre randomised Phase II clinical trial for the indication of advanced stage colorectal cancer [101]. Current standard of care, 5-fluorouracil/leucovorin (FU/LV), was compared to treatment with FU/LV plus LD BV (5 mg/kg every 2 weeks) or with HD BV (10 mg/kg every 2 weeks). This study also demonstrated increases in median time prior to progression and overall response rate in patients treated with BV. This improvement reached statistical significance with the addition of LD but not HD BV. Compliance with BV therapy was quite good and patients receiving BV were more likely to receive all planned cycles of chemotherapy and to stay on therapy far longer than controls. Amongst the BV containing regimens, there was an increased incidence of hypertension, gastrointestinal haemorrhage, epistaxis and proteinuria which was consistent with previous trials. There was also a trend toward an increased number and severity of thromboembolic complications with BV. Specifically, there were three events on the control arm, as compared with nine on the LD and four on the HD BV arm, with one resultant fatality; but here again, the results suggest that the clinical benefit outweighs the additive risk.

Several explanations for the unexpected finding that LD BV therapy was superior to HD BV were offered, including the limitations of this type of study: chance and the possible over-representation of poor prognosis patients in the high-dose arm. Nonetheless, the alternative hypothesis, that lower levels of VEGF blockade are more effective than higher doses in colon cancer, is also intriguing. The authors suggest that BV doses which are antiangiogenic but prevent total vascular collapse may, in fact, be more tumouricidal, due to improved delivery of chemotherapy. Further studies will need to clarify whether different tumours require different optimal dosing. This study has formed the basis of two Phase III randomised, placebo-controlled studies of BV at 5 mg/kg with FU/LV, with or without irinotecan, for > 900 patients with metastatic colorectal cancer.

4.2 HuMV833

Another humanised VEGF neutralising antibody, HuMV833 (Toagosei Co. Ltd/Protein Design Labs, Inc.), has recently entered into clinical trials in Europe. This agent recognises two of the VEGF isoforms and utilises the human IgG4k backbone. Initial preclinical and clinical experience suggests a similar toxicity profile to BV. In a Phase I study designed to determine the optimum biologically active dose using novel imaging techniques, the investigators demonstrated the inherent difficulty in determining the standard dose for antiangiogenic agents versus cytotoxic drugs [102]. By positron emission tomography (PET) pharmacodynamic study, they have shown that, even within tumour deposits of the same patient, drug distribution and clearance can vary greatly after intravenous infusion. This same intratumoural variability was seen when vascular permeability was measured using dynamic enhanced magnetic resonance imaging (DEMRI). They suggest future trials might incorporate a second level of Phase I testing in which functional imaging is used to follow a single cohort with intra-patient dose escalation. Alternatively, larger cohorts with similar clinical features could be used in order to generate more accurate response data.

4.3 VEGF-Trap

VEGF-Trap (Regeneron Pharmaceuticals, Inc.) is a novel chimaeric fusion molecule, consisting of the second Ig domain of VEGFR-1 combined with the third domain of VEGFR-2, fused to the Fc portion of IgG1 [76]. By using portions of two receptors, VEGF-Trap has markedly improved pharmacokinetics but retains high-affinity binding to all isoforms of human VEGF. Since VEGF-Trap is composed entirely of human sequences, it is anticipated that this will minimise the possibility of immunogenic responses in human patients. VEGF-Trap irreversibly binds free VEGF, thus preventing ligand binding to its receptors. VEGF-Trap inhibits tumour growth of a variety of different tumour types including rhabdomyosarcoma, glioma, melanoma and neuroblastoma [76,103]. It has also been shown that treatment with VEGF-Trap not only results in virtually avascular

tumours but can also cause regression of coopted vasculature, unlike anti-VEGF antibody [103].

VEGF-Trap has several potential advantages over monoclonal neutralising antibody: it has a significantly higher affinity (1 – 5 pM) to VEGF; it has excellent bioavailability after subcutaneous injection, with preclinical pharmacokinetics suggesting that weekly administration of VEGF-Trap should be adequate in human clinical trials; and lastly, VEGF-Trap has the ability to bind other VEGF family members, such as PlGF [76]. Recently, the first Phase I dose escalation trial with pharmacokinetics of VEGF-Trap has begun accrual. Patients are treated with a single subcutaneous dose at week one, followed by a 28-day washout and then one dose per week for 6 weeks [104]. Patients have received drug at dose levels of 25 and 50 µg/kg. Preliminary data suggests that VEGF-Trap complexes to circulating VEGF in plasma and that no anti-VEGF-Trap antibodies have been detected in any of the patients treated.

4.4 IMC-1C11

IMC-1C11 (ImClone Systems) is a high-affinity ($K_d = 0.8$ nM) chimaeric mouse/human antihuman antibody recognising VEGFR-2, blocking VEGF ligand interaction and subsequent signal transduction [105,106]. This IgG1 antibody utilises a single chain antibody (scFv) which binds to an epitope within the VEGF recognition site on the extracellular N-terminus of VEGFR-2. Preclinical testing has been done primarily with the related antibody DC-101, since IMC-1C11 is specific for the human VEGFR-2. Multiple studies with DC-101 have demonstrated preclinical efficacy in a variety of tumours [78,107]. Klement *et al.* have also demonstrated that combination therapy with LD vinblastine and DC-101 antibody results in full and sustained regression of large established tumours, without any signs of acquired drug resistance for 180 days [108]. This, and a paper by Browder *et al.* [109], were the first to demonstrate the concept of antiangiogenic or metronome scheduling of cytotoxic agents [110]. Recently, IMC-1C11 has shown potent activity against human leukaemia xenografts [13,61]. IMC-1C11 has recently entered clinical trials, with the Phase I trial reported on 14 patients with advanced colorectal cancer with liver metastasis [111]. Single agent IMC-1C11 was delivered as a weekly infusion at doses ranging 0.2 – 4.0 mg/kg and was well-tolerated with no CTC grade 3 – 4 infusion related toxicities.

4.5 SU5416, SU6668 and SU11248

Scientists at SUGEN (now Pharmacia) were among the first to develop small molecular inhibitors of protein tyrosine kinases, of which SU5416 is the prototype [79,112,113]. These small molecular inhibitors interfere with the ATP-binding site of the receptor kinase, thus preventing autophosphorylation of the receptor in response to ligand binding [112]. Although, there is some receptor specificity, these molecules will generally inhibit families of receptors. SU5416 inhibits the VEGFR-1, VEGFR-2, VEGFR-3, c-kit and flt-3 receptors [79]. SU6668 has a similar spectrum of activity,

however, with increased inhibition of platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) receptors [114]. *In vivo* antitumour activity of SU5416 and SU6668 has been demonstrated in numerous murine and human tumour cell line implant studies, including melanoma, glioma, fibrosarcoma, neurogenic sarcoma, and lung, mammary and prostate carcinomas [79,113-116]. SU6668 may be more potent than SU5416 by virtue of its ability to inhibit PDGF receptor (PDGFR) and thus disrupt interaction of surrounding stromal cells, such as pericytes, with vascular endothelial cells [114,116].

SU5416 has undergone relatively extensive clinical testing. This initially promising agent displayed a number of adverse characteristics, including general toxicity, unfavourable pharmacokinetics and a unique and unexpected series of thromboembolic events when given in combination with cisplatin and gemcitabine. After failing to demonstrate significant benefit over chemotherapy alone in early results of a Phase III trial for metastatic colorectal cancer, the company eventually chose not to pursue further development of this agent [202].

Nonetheless, a review of the completed clinical trials is highly informative. Numerous Phase I dose escalation trials were undertaken in order to establish a tolerable schedule and dose capable of generating clinically relevant drug levels. Two trials studied twice-weekly intravenous infusions in patients with advanced malignancies at doses ranging 4.4 – 190 mg/m². Side effects included hypersensitivity to the diluent (cremophor), phlebitis, other local reactions, transaminitis, and significant nausea, vomiting and headache, which were dose-limiting in one study. The recommended dose for Phase II trials on this schedule was 145 mg/m², with dexamethasone use as a premedication. Neither trial could report an objective tumour response, but 13 of the combined 84 patients showed stabilisation of disease, and reductions in tumour vascular permeability could be demonstrated by DEMRI [117,118]. A clear stumbling block was identified as rapid and inducible clearance of the agent with repeat dosing [119].

A more recent Phase I trial examined the feasibility of a 5-day load (20 mg/m²/day) followed by weekly infusion (range, 65 – 190 mg/m²) [120]. This study showed significantly different pharmacokinetics than the previous studies, highlighting the importance of dose schedule. Pharmacokinetic data revealed that the weekly infusion schedule prevented the reported 50 – 60% induction in SU5416 clearance observed with either daily or twice-weekly dosing. Coincident with higher areas under the curve, was increased toxicity with grade 3 headache, hypersensitivity and reversible acute renal failure. Of the four patients experiencing clinical benefit, at least two discontinued therapy due to toxicity [120].

In Phase II trials, > 650 patients received SU5416 on the twice-weekly schedule, with or without cytotoxic agents. As a single agent, exciting potential activity was seen, predominantly in advanced colorectal disease, but also in mesothelioma, refractory Kaposi's sarcoma, head and neck cancers,

melanoma, acute myelogenous leukaemia and, possibly, renal cell carcinoma [120-122]. In combination with cytotoxic agents, the pharmacokinetics of SU5416 were similar to those of the drug given as a single agent. There were objective responses, stabilisation of disease and what appeared to be a prolonged median survival time [117,123,124].

However, of particular interest and concern were the results of a Phase I trial of SU5416 in combination with cisplatin and gemcitabine for refractory solid tumours. The study required early stopping after nine vascular events, including transient ischaemic attack (TIA), cerebral vascular accident (CVA), DVT and pulmonary embolus, were seen in 8 of 19 patients. Given the tendency toward bleeding seen in trials with BV, recommendations for anticoagulation were controversial. Pharmacokinetics did not support a specific drug interaction, but a review of previous trials supported a peculiar tendency towards endothelial injury with this combination [125]. Subsequent analysis suggests that deprivation of VEGF signalling by SU5416, in the context of this regimen, activates endothelium, as evidenced by increased levels of von Willebrand factor, soluble endothelial selectin (E-selectin) and soluble tissue factor. If so, this unfortunate clinical outcome provides further evidence that VEGF is critical in the stabilisation of quiescent endothelial cells [126].

The broader spectrum SU6668 is orally bioavailable and thus has potential for chronic therapy. However, clinical utility is hampered by a short plasma half-life of ~ 1 h and inducible clearance, as with SU5416. To date, clinical trials have been restricted to Phase I single agent dose escalation studies. Patients have been treated 1 – 3 times daily in 28-day cycles. Once-daily dosing at relatively high doses was well-tolerated, but target plasma levels ($> 2.3 \mu\text{g/ml}$) were achieved for < 10 h/day. Twice and three times daily doses were less well-tolerated but could achieve a more clinically relevant area under the curve. Frequent grade 1 – 2 toxicities included abdominal pain, nausea and fatigue. Dose-limiting grade 3 toxicities of atypical chest pain associated with dyspnea and serositis were observed. Phase II trials are proceeding using doses 300 mg/m^2 b.i.d. or 100 mg/m^2 t.i.d. Clinical data are preliminary but there are several reported cases of disease stabilisation lasting > 6 months. [127-131]. It remains to be seen whether the effect of SU6668 on a broader range of receptor kinases with angiogenic roles increases efficacy or toxicity, particularly if given in combination with cytotoxic agents.

Also in development within this family of small molecules is SU11248, which blocks VEGFR-1, VEGFR-2, PDGFR, flt3 and c-kit [132,133]. Therefore, the drug may have primary antiproliferative activity in addition to its antiangiogenic properties. Exciting preliminary data suggests this oral agent is active in nanomolar concentrations, which can be achieved for > 12 h with daily or alternate day dosing. A dose escalation trial is ongoing and objective clinical responses in refractory solid tumours have been reported [133]. Early drug-related toxicity seems to be limited to oedema, weakness,

thrombocytopenia, neutropenia, diarrhoea, mucositis and discolouration of the skin and hair. The peculiar challenge of this agent may again be tumour necrosis, which has caused peritonitis and death in one subject [133].

4.6 Other orally-active agents

PTK787/ZK222584 (vatalanib; Novartis/Schering AG) is another orally-active, small molecule inhibitor of the VEGF family receptors [81,134]. Like SU6668, it also blocks the PDGFR and c-kit tyrosine kinases by competitive binding to the ATP docking site. It has been shown to inhibit a wide variety of tumours in preclinical models, including renal cell carcinoma [134] and myeloma [135], to inhibit pleural effusions from lung adenocarcinoma [136] and to increase the efficacy of radiation therapy [137]. Vatalanib has entered Phase I clinical trials in Europe and the US, predominantly in patients with advanced colorectal cancer and glioblastoma. In three separate Phase I trials, using doses ranging $50 - 2000 \text{ mg/m}^2$ once-daily, there have been no dose-limiting toxicities. The terminal half-life is somewhat favourable at 3 – 6 h. Common side effects were fatigue, nausea and vomiting, light-headedness and ataxia, transaminase elevation and further elevation of blood pressure in patients with pre-existing hypertension. Having benefited from prior experience, these trials were designed to identify surrogate markers for biologic activity in addition to clinical response. These studies are promising, not only in that they show a significant number of patients achieving stabilisation of disease, but also because reduction of vascular permeability, as measured by DEMRI, and elevations of serum VEGF and basic FGF (bFGF) seemed to correlate with clinical non-progression [138-140].

ZD6474 (AstraZeneca) is also beginning Phase I trials in refractory solid tumours. ZD6474 is an orally-active small molecular inhibitor that blocks VEGFR-2, VEGFR-1 and, to some extent, EGFR tyrosine kinases [80] and oncogenic RET kinases [141]. Significant tumour suppression was seen with oral administration in preclinical models of human lung, prostate, breast, ovarian, colon and vulval cancers [80]. This agent has a particularly favourable pharmacokinetic profile and seems well-tolerated, with side effects of rash, diarrhoea and asymptomatic prolongation of corrected QT interval reported to date [142-144].

CP-547,632 (Pfizer) is a potent orally-active VEGFR-2 inhibitor and has shown efficacy against a variety of human xenografts in nude mice (NSCLC, colon, breast) [145]. In a Phase I study of patients with advanced solid tumours, dose-limiting toxicity has not been observed and preliminary safety data reveal no serious adverse events or study discontinuations related to CP-547,632 therapy [146]. Of the 22 patients evaluable for tumour response, 6 patients have stable disease beyond 2 courses of treatment (> 8 weeks) and 1 patient has stable disease beyond 6 courses (> 24 weeks).

4.7 Angiozyme

Angiozyme is a member of a new designer class of drugs called chemically stabilised ribozymes, which are synthesised

to target, bind and cleave a specific mRNA sequence. It is the first such agent to enter human trials, and it works by targeting the mRNA for VEGFR-1 [147]. Angiozyme was chosen over anti-VEGFR-2 ribozymes because, although both can inhibit primary tumour growth in a Lewis lung carcinoma model, only Angiozyme significantly inhibits lung metastasis [147].

A clinical trial designed to test Angiozyme's safety, tolerability and pharmacokinetics profile in healthy volunteers has been reported [148]. Angiozyme administration was well-tolerated, with minor side effects of headache and somnolence reported. An average elimination half-life of 28 – 40 min was observed after intravenous administration, which increased to 209 min after subcutaneous administration. Further clinical testing of Angiozyme in patients with a history of cancer, has shown that Angiozyme is well-tolerated by both routes of administration and has maintained plasma levels for prolonged periods, up to 24 h when delivered by the subcutaneous route [149]. Currently, there are Phase I/II trials for Angiozyme in combination with cytotoxic therapy for the lead indications of breast and colorectal cancer, with preliminary evidence indicating that it is well-tolerated and may improve time-to-progression [150].

5. Conclusion and expert opinion

It is too early to know which VEGF blockade therapy will prove the most effective and in which tumours this efficacy will be demonstrated. Each agent and class of agents appears to offer distinct advantages and disadvantages. Antibodies to VEGF or decoy receptors like VEGF-Trap have the benefit of being highly specific for VEGF, and thus may have limited toxicity that is unrelated to VEGF blockade, in contrast to small molecular inhibitors which may inhibit a wide variety of tyrosine kinases besides VEGFR. In some instances, however, a broader spectrum of inhibition may be useful. For example, SU6668, by inhibiting both the VEGF and PDGF pathways, results in rapid apoptosis of tumour vasculature and tumour regression [116]. The small molecular inhibitors also offer the advantage for chronic therapy of being orally-active.

From complex analysis of the potential of these therapies, it is clear that not all experimental tumours are equivalently, or even effectively, suppressed by VEGF antagonism. It has been shown in murine xenograft models that while anti-VEGF antibody therapy almost totally suppresses Wilms' tumour growth [91], it only partially suppresses neuroblastoma [151,152] and is ineffective against rhabdoid tumour [153]. This is despite VEGF being expressed in all three tumour types. Therefore, it may be difficult to predict whether a particular type of tumour, let alone an individual patient's cancer, will respond to VEGF inhibition. Indeed, the correlative studies on VEGF levels from various clinical trials have been unable to reliably predict which patients will respond to VEGF blockade therapy.

The issue of how best to measure response in a non-cytotoxic agent, where clinical non-progression is likely evidence

of potent activity, has not been resolved. Additionally, when determining optimal dosing, biological end points such as lack of free VEGF may be more relevant than toxicity. However, the appropriate biological end point for effective anti-VEGF blockade is not yet clear. Measurements of circulating free VEGF may be subject to biologically relevant conditions *in vivo*, such as tissue hypoxia and activation of inflammatory cascades, as well as confounding methodological inconsistencies in the laboratory [154]. It is also possible that while free serum VEGF may be absent, it does not accurately reflect locally produced VEGF by either tumour or associated stromal cells. A variety of other surrogate markers for angiogenesis have been proposed. These include bFGF, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and von Willebrand's factor antigen [155-157]. The role of these markers in assessment of patient response remains an interesting avenue of investigation.

In addition, the timing of VEGF blockade therapy in a patient's regimen remains a major problem. Theoretically, antiangiogenic agents might be most efficacious in treating small tumours or those prior to metastatic spread, rather than treating bulky tumours or rapidly progressing tumours; yet virtually all the clinical trials with VEGF blockers have been in patients with relapsed, bulky disease. An inability to inhibit rapidly progressive tumours may explain why the Phase III trial for women with pretreated metastatic breast cancer, use of BV and capecitabine failed to show an improvement in overall response rate compared to capecitabine alone [203]. Long-term therapy has been shown to be of benefit in suppressing tumour growth [96]. Therefore, chronic or maintenance therapy, particularly in the case of minimal residual disease, may be where VEGF blockade is most effective. This strategy awaits clinical testing.

Since VEGF blockade therapy appears unlikely to be effective as monotherapy, another major obstacle is to determine how best to combine these agents with chemotherapy, radiation or other biological modifiers, from both an efficacy and toxicity standpoint. Should these compounds be given as part of standard chemotherapy regimens [101] or should they be given with agents for which they might work synergistically, as suggested by the metronomic dosing experiments [108,158]? The heightened risks in combination therapy, as highlighted by the trials with SU5416, may include vascular-related events such as thromboembolism [125].

Lastly, despite the initial hope that antiangiogenic therapy would not elicit resistance [159], there is accumulating evidence that tumours can resume growth after an initial response. It is possible that some tumours may become resistant to therapy by virtue of expressing other angiogenic factors [160] or by coopting existing vasculature [161]. Potential counter-strategies could involve combined use with inhibitors to these other factors (e.g., STI-571 to inhibit PDGFR) or using a more potent antagonist (e.g., VEGF-Trap regresses coopted vessels [103]).

Yu *et al.* have reported that mice bearing tumours derived from p53^{-/-} HCT116 human colorectal cancer cells were less responsive to antiangiogenic combination therapy than mice bearing isogenic p53^{+/+} tumours [162]. These results demonstrate that genetic alterations that decrease the vascular dependence of tumour cells can influence the therapeutic response of tumours to this therapy.

Despite the significant obstacles outlined above, one should be optimistic that VEGF blockade holds great promise for cancer therapy. Selection of tumours that are sensitive, determining the right dosing, setting and combination therapy, and understanding the basis of therapeutic resistance will permit the optimal use of anti-VEGF agents, and are the challenges for the future.

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